Multigene phylogenetic analysis of the *Trichomonascus*, *Wickerhamiella* and *Zygoascus* yeast clades, and the proposal of *Sugiyamaella* gen. nov. and 14 new species combinations

Cletus P. Kurtzman & Christie J. Robnett

Microbial Genomics and Bioprocessing Research Unit, National Center for Agricultural Utilization Research, Agricultural Research Service, U. S. Department of Agriculture, Peoria, IL, USA

Downloaded from https://academic.oup.com/femsyr/article/7/1/141/492833 by The University of Texas at Austin user on 03 July 2024

Correspondence: Cletus P. Kurtzman, Microbial Genomics and Bioprocessing Research Unit, National Center for Agricultural Utilization Research, Agricultural Research Service, U. S. Department of Agriculture, 1815 North University Street, Peoria, IL 61604, USA. Tel.: +1309 681 6561; fax: +1309 681 6672; e-mail: kurtzman@ncaur.usda.gov

Received 19 April 2006; revised 31 May 2006; accepted 2 June 2006.
First published online 12 September 2006.

DOI:10.1111/j.1567-1364.2006.00157.x

Editor: Teun Boekhout

Keywords
yeasts; multigene phylogeny; *Arxula*; *Blastobotrys*; *Sympodiomyces*; *Trichomonascus*.

Abstract

Relationships among species assigned to the ascosporic yeast genera *Sporopachydermia*, *Stephanoascus*, *Trichomonascus*, *Wickerhamiella* and *Zygoascus*, and to the associated anamorphic genera *Arxula*, *Blastobotrys*, *Sympodiomyces* and *Trigonopsis*, were determined from phylogenetic analyses of gene sequences from the nearly complete large-subunit rRNA gene, the mitochondrial small-subunit rRNA gene, and cytochrome oxidase II. The genus *Stephanoascus* is polyphyletic, resulting in reassignment of two species to the older genus *Trichomonascus* and the third to *Sugiyamaella* gen. nov. (type species *Sugiyamaella smithiae*). The genera *Sporopachydermia*, *Wickerhamiella* and *Zygoascus* appear to be monophyletic. The species *Pichia ofunaensis* and *P. tannicola* are proposed for transfer to *Zygoascus*. *Arxula*, *Blastobotrys* and *Sympodiomyces* are members of the *Trichomonascus* clade, with the genus *Blastobotrys* having taxonomic priority for anamorphic states. *Trigonopsis variabilis* and three species of *Candida* represent a distinct clade. From the foregoing gene sequence analyses, the new ascosporic genus *Sugiyamaella* is proposed, as are 14 new species combinations and the new family *Trichomonascaceae*.

Introduction

Phylogenetic analysis of domains D1 and D2 (D1/D2) of large-subunit (26S) rRNA genes have shown that species of the ascosporic genera *Stephanoascus*, *Wickerhamiella* and *Zygoascus* are members of the same large clade (Kurtzman & Robnett 1995, 1998). Included in this clade were the anamorphic genera *Arxula*, *Blastobotrys* and *Sympodiomyces*, some species assigned to *Candida*, and possibly the genus *Trigonopsis*. Because deep lineages are seldom well resolved from phylogenetic analysis of a single gene, evolutionary relationships among the preceding genera were unclear.

In the present study, we have analyzed members of this large clade from gene sequences of the nearly entire large-subunit rRNA gene, mitochondrial small-subunit rRNA gene and cytochrome oxidase II, and analysis of the combined gene sequences has provided much greater phylogenetic resolution than obtained from the earlier D1/D2 rRNA gene studies. Furthermore, inclusion of 21 new species in this clade has provided added clarification of relationships. The new species included in the present analysis will be formally described in subsequent reports. An additional aspect of this work was the discovery of up to six introns in the large-subunit rRNA gene sequences of certain species. Intron evolution, as assessed from phylogenetic relationships among species, will be the subject of a future report. In the present study, phylogenetic circumscription of genera has been examined with multigene analysis, and the results have led to the proposal of a new ascosporic genus and 14 new combinations of described species that are presently assigned to other genera.

Materials and methods

Organisms

The strains studied are listed in Table 1, and all are maintained in the ARS Culture Collection (NRRL), National Center for Agricultural Utilization Research, Peoria, IL, USA.

© 2006 Federation of European Microbiological Societies
Published by Blackwell Publishing Ltd. No claim to original US government works

LCY Biotechnology Holding, Inc.
Ex. 1025
Page 1 of 11

Table 1. Yeast strains compared in this study

Species	Strain designation NRRL	Strain designation CBS	Species	Strain designation NRRL	Strain designation CBS
Arxula adeninivorans	Y-17692^T	8244	*C. vanderwaltii*	Y-17671^T	5524
A. terrestris	Y-17704^T	7376	*C. versatilis*	Y-6652^T	1752
Blastobotrys arbuscula	Y-17585^T	227.83	*C. vinaria*	Y-5715^T	4077
Bla. aristata	Y-17579^T	521.75	*Candida* sp. n.	Y-17858^T	7922
Bla. capitulata	Y-17573^T	287.82	*Candida* sp. n.	Y-27140^T	6663
Bla. elegans	Y-17572^T	530.83	*Candida* sp. n.	Y-27117^T	5924
Bla. nivea	Y-17581^T	163.67	*Candida* sp. n.	Y-27161^T	7317
Bla. proliferans	Y-17577^T	522.75	*Candida* sp. n.	YB-1336^T	10341
Blastobotrys sp. n.	Y-6417^T	10336	*Candida* sp. n.	YB-1473^T	10342
Blastobotrys sp. n.	Y-6844^T	10337	*Candida* sp. n.	YB-1835^T	10344
Blastobotrys sp. n.	Y-7993^T	10338	*Candida* sp. n.	YB-1847^T	10346
Blastobotrys sp. n.	Y-27150^T	6800	*Candida* sp. n.	YB-2263^T	10348
Blastobotrys sp. n.	YB-1343^T	10339	*Candida* sp. n.	YB-2450^T	10349
Blastobotrys sp. n.	YB-2290^T	10340	*Candida* sp. n.	YB-3827^T	10350
Botryozyma nematodophila	Y-17705^T	7426	*Pichia ofunaensis*	Y-10998^T	8129
Candida auringiensis	Y-17674^T	6913	*P. tannicola*	Y-17392^T	6065
C. azyma	Y-17067^T	6826	*Sporopachydermia cereana*	Y-7798^T	6644
C. bertae var. *bertae*	Y-17643^T	8169	*Sp. lactativora*	Y-11591^T	6192
C. blankii	Y-17068^T	1898	*Sp. quercuum*	Y-17847^T	8070
C. cantarellii	Y-17650^T	4878	*Stephanoascus ciferrii*	Y-10943^T	5295
C. caseinolytica	Y-17796^T	7881	*St. farinosus*	Y-17593^T	140.71
C. castrensis	Y-17329^T	8172	*St. smithiae*	Y-17850^I	7522.2
C. chiropterorum	Y-17071^T	6064	*Sugiyamaella* sp. n.	YB-2067^T	10352
C. drosophilae	Y-27366^T	8459	*Sugiyamaella* sp. n	YB-2798	
C. galacta	Y-17645^T	6939	*Sympodiomyces attinorum*	Y-27639^T	9734
C. ghanaensis	YB-1486^T	8798	*Sym. indianaensis*	YB-1950^T	9600
C. litsaeae	YB-3246^T	8799	*Sym. parvus*	Y-10004^T	6147
C. mokoenaii	Y-27120^T	8435	*Trichomonascus petasosporus*	YB-2092^T	9602
C. novakii	Y-27346^T	8402	*Trigonopsis variabilis*	Y-1579^T	1040
C. ontarioensis	YB-1246^T	8502	*Trigonopsis* sp. n.	Y-27307^T	10351
C. paludigena	Y-12697^T	8005	*Wickerhamiella australiensis*	Y-27360^T	8456
C. pararugosa	Y-17089^T	1010	*W. cacticola*	Y-27362^T	8454
C. petrohuensis	Y-17663^T	8173	*W. domercqiae*	Y-6692^T	4351
C. salmanticensis	Y-17090^T	5121		Y-6698	4733
C. santjacobensis	Y-17667^T	8183	*W. lipophila*	Y-27367^T	8458
C. sorbophila	Y-7921^T	6739	*W. occidentalis*	Y-27364^T	8452
C. spandovensis	Y-17761^T	6875	*Zygoascus hellenicus*	Y-7136^T	5839
C. tartarivorans	Y-27291^T	7955		Y-27156	4028
C. tepae	Y-17670^T	5115	*Z. meyerae*	Y-17319^T	4099
C. valdiviana	Y-7791^T	5721	*Schizosaccharomyces pombe*	Y-12796^T	356

NRRL, ARS Culture Collection, National Center for Agricultural Utilization Research, Peoria, IL, USA; CBS, Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; T, type strain; I, isotype strain.

Growth of cultures and DNA isolation

Methods for the growth of cultures and extraction of DNA have been presented in detail by Kurtzman & Robnett (1998). Briefly, cultures were grown for 24–48 h in YM broth (Yarrow, 1998a) and harvested by centrifugation. Cells were freeze-dried for 1–2 days, and the dried cells were then broken by shaking with 0.5-mm glass beads. DNA was extracted from the fractured cells using either a sodium dodecyl sulfate–phenol/chloroform protocol or through use of CTAB/chloroform.

DNA sequencing

Amplicons of the three genes sequenced were synthesized by PCR using the primer pairs and conditions listed below. Symmetrical amplification and sequencing reactions were conducted using a 96-well plate format. Amplicons were purified from PCR reactions using Millipore Multiscreen PCR plates (Billerica, MA). Both DNA strands of the genes were sequenced with the ABI TaqDyeDeoxy Terminator Cycle sequencing kit (Applied Biosystems, Foster City, CA) using either ABI 3100 or ABI 3730 automated DNA

Downloaded from https://academic.oup.com/femsyr/article/7/1/141/492833 by The University of Texas at Austin user on 03 July 2024

© 2006 Federation of European Microbiological Societies
Published by Blackwell Publishing Ltd. No claim to original US government works

LCY Biotechnology Holding, Inc.
Ex. 1025
Page 2 of 11

sequencers, following the manufacturers' instructions. Prior to sequencing, DNA fragments from the TaqDyeDeoxy sequencing reactions were recovered by precipitating with 75% ethanol.

Mitochondrial small-subunit rRNA gene

Primers for symmetrical amplification of the gene, and the subsequent sequencing reactions, included the primers given earlier by Kurtzman & Robnett (2003), as well as various combinations of the following primers: ARXIOMS-1F (5′-TAATTGTGCCAGCAGTCGCGG), ARXIOMS-2R (5′-CGTGCTCCACTACTTAAGTCTG), MS-BLA-1F (5′-GGYHTAAAGVRTYAGYAR), MS-BLA-2R (5′-ATTAAA-TAACATRMTCCACTG), and MS-BLA-2AR (5′-CBGYC-TAWTGTYTTRRRTTTC). Temperatures for symmetrical amplification were denaturation at 96 °C and either 42 °C for annealing and 45 °C for extension or 39 °C for annealing and 45 °C for extension. For a few of the sequencing reactions, 42 °C for annealing and 45 °C for extension were used, in contrast to the standard temperatures of 50 °C/60 °C.

Cytochrome oxidase II

The various primer combinations used for symmetrical amplification and gene sequencing were those reported earlier (Kurtzman & Robnett, 2003), as well as the following, which were used in various combinations: COII-5C (5′-GTTCTATATCTTATTAATCG), COII-5E (5′-GTWTTATW TRRTAWTARTAWTATG), COII-5F (5′-SWTATAAATATT-TARTWCATGG), COII-3C (5′-CTTGATTTAATCTACCAG GATTAGC), COII-3E (5′-CCACATAWTTCWBDACAYTK WCC), COII-3F (5′-CCTTCWCYTTGWATWAWWGTAC). Temperatures for symmetrical amplification were denaturation at 96 °C and either 45 °C for annealing and 72 °C for extension or 39 °C for annealing and 60 °C for extension. For the sequencing reactions, annealing was at 42 °C and extension at 45 °C.

Large-subunit rRNA gene

Many of the species in this study had one to six introns of *c.* 400–1200 bp inserted in the large-subunit (LSU) rRNA gene, often at the conserved, commonly used priming sites. The presence of introns increased the length of the LSU gene by up to 5.5 kb for some species, and the usual strategy of amplifying the LSU in two overlapping halves often failed because of the presence of introns in priming sites. Consequently, the amplicons used for sequencing varied among species, but were generated with the primers listed by Kurtzman & Robnett (2003), as well as the following, which are listed sequentially (5′–3′) across the LSU gene: NL-6F (5′-CTTGTTACTTAATTGAACGTGGAC), NL-6R (5′-GTCCACGTTCAATTAAGTAACAAG), NL-7F (5′-CATC-TAAACAGCCGGACGGTGGC), NL-7BF (5′-GACAGCCG-GACGGTGGCCATGGAAGTCG), NL-7CF (5′-GTGTAAC AACTCACCGGCCGAATG), NL-7CR (5′-CATTCGGCC GGTGAGTTGTTACAC), NL-7DF (5′-GCCTCTAGTGCA-GATCTTGGTGGTAGTAG), NL-7DR (5′-CTACTACCAC-CAAGATCTGCACTAGAGGC), NL-1611BF (5′-CGCAGCA GGTCTCCAAGGTKAACAGC), NL-11AR (5′-CAGTCA-GATTCCCCTTGTCCGTAC), NL-11BF (5′-CTGACTGTC-TAATTAAAACATAGC), NL-12AF (5′-CTATCTAGCGAA ACCACAGC), NL-12AR (5′-GCTGTGGTTTCGCTAGA-TAG), NL-15F (5′-CATGAAAGTGTGGCCTATCGATC), NL-15R (5′-GATCGATAGGCCACACTTTCATG), NL-G19BR (5′-CTAACCTGTCTCACGACGGTC), NL-G19CF (5′-GCAGTCAAGCGTTCATAGCG), NL-G19DF (5′-CAG GGATAACTGGCTTGTGGCAGTC), NL-G19DR (5′-GAC TGCCACAAGCCAGTTATCCCTG), NL-G19ER (5′-GATA GGAAGAGCCGACATCGAAG), NL-STB1IF (5′-GAAAC TCTGGTGGAGGCTCGTAG), NL-E27AF (5′-CTTAAGG-TAGCCAAATGCCTCGTCATC), NL-E27AR (5′-GATGAC-GAGGCATTTGGCTACCTTAAG), NL-E27BF (5′-GGATTA ACGAGATTCCCACTG), NL-E27BR (5′-CAGTGGGAAT CTCGTTAATCC), NL-E27DF (5′-CTCATGGAGAACA-GAAATCTCC), NL-E27DR (5′-GGAGATTTCTGTTCTC-CATGAG), NL-ETS2-1AR (5′-GGCTTAATCTCAGCAGA TCG), NL-ETS2-GR (5′-GATCGTAACAACAAGGCTACT CTACTG), and NL-ETS2-IR (5′-GGATTCTGACTTAG AGGCGTTCAG). Temperatures for symmetrical amplification were 96 °C for denaturation, 52 °C for annealing, and 72 °C for extension.

Phylogenetic analysis

Phylogenetic relatedness among taxa was determined from the maximum parsimony and neighbor-joining programs of PAUP* 4.063a (Swofford, 1998). The Kimura-2 parameter distance correction was used for neighbor-joining analyses. Bootstrap support for all phylogenetic trees was determined from 1000 replications. Introns and regions of uncertain nucleotide alignment were excluded from phylogenetic analysis.

Results and discussion

Phylogenetic analyses

Trees derived from phylogenetic analysis of the LSU rRNA gene (Fig. 1), a combined dataset of the mitochondrial small-subunit (MtSm) rRNA gene with cytochrome oxidase II (COXII) (Fig. 2) and a concatenation of all three gene sequences (Fig. 3) illustrate the extent of resolution derived from each of these datasets. Both the LSU gene sequence and the combined MtSm–COXII sequences provided similar resolution of taxa, with some basal lineages ending in

Downloaded from https://academic.oup.com/femsyr/article/7/1/141/492833 by The University of Texas at Austin user on 03 July 2024

© 2006 Federation of European Microbiological Societies
Published by Blackwell Publishing Ltd. No claim to original US government works

LCY Biotechnology Holding, Inc.
Ex. 1025
Page 3 of 11

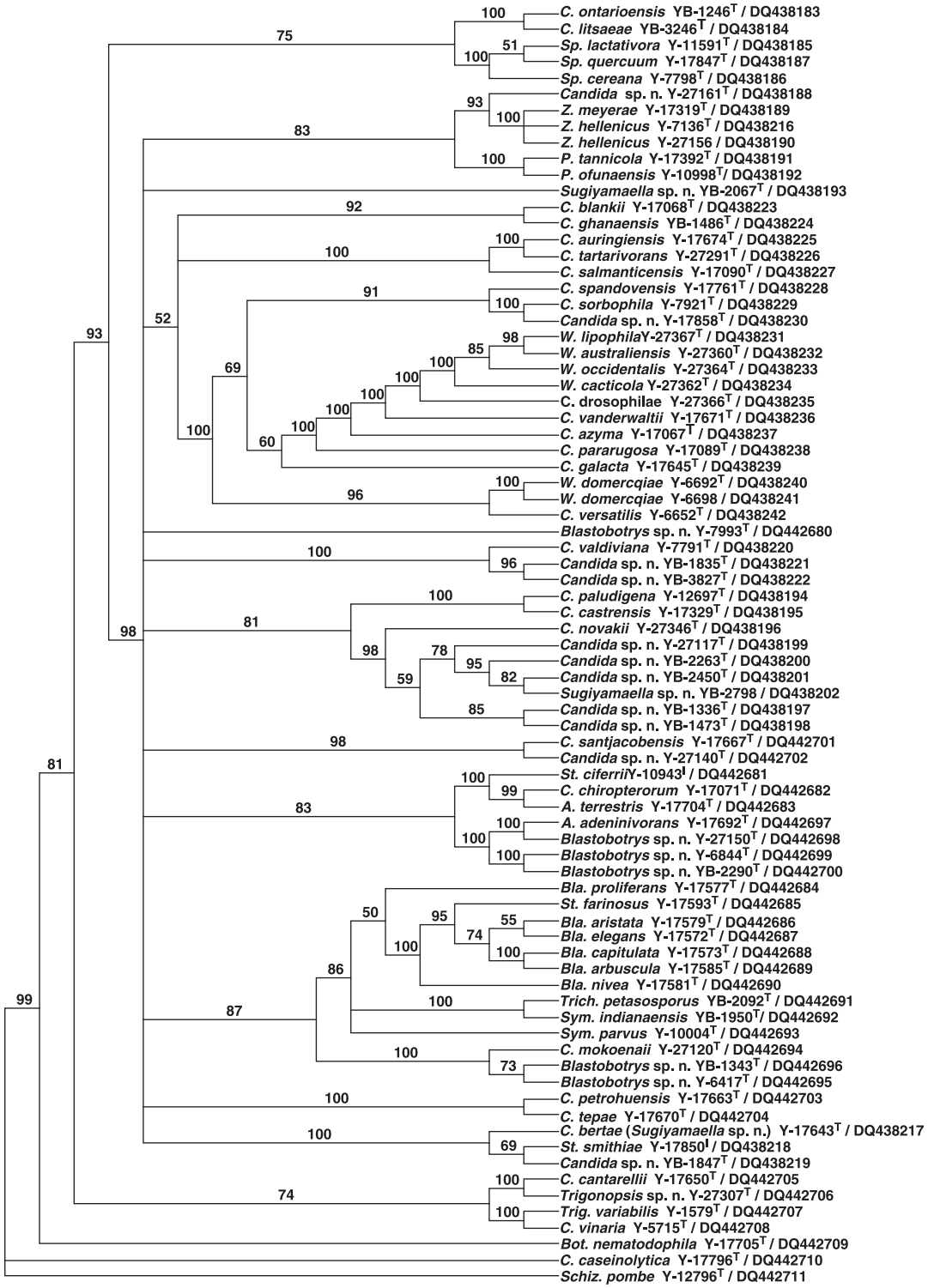

Fig. 1. Bootstrap consensus tree of species in the *Trichomonascus–Wickerhamiella–Zygoascus* species complex from maximum parsimony analysis of 26S rRNA genes, which gave 12 most parsimonious trees. Bootstrap values ≥50% are given at branch nodes based on 1000 replications. Tree length = 3936, consistency index (CI) = 0.417, retention index (RI) = 0.718, rescaled consistency index (RC) = 0.300, parsimony-informative characters = 818. *Schizosaccharomyces pombe* was the designated outgroup species for the analysis. Abbreviations for all figures: *A.*, *Arxula*; *Bla.*, *Blastobotrys*; *Bot.*, *Botryozyma*; *C.*, *Candida*; *P.*, *Pichia*; *Schiz.*, *Schizosaccharomyces*; *Sp.*, *Sporopachydermia*; *St.*, *Stephanoascus*; *Sym.*, *Sympodiomyces*; *Trich.*, *Trichomonascus*; *Trig.*, *Trigonopsis*; *W.*, *Wickerhamiella*; *Z.*, *Zygoascus*; T, type strain; I, isotype strain. GenBank accession numbers follow strain designations.

Downloaded from https://academic.oup.com/femsyr/article/7/1/141/492833 by The University of Texas at Austin user on 03 July 2024

© 2006 Federation of European Microbiological Societies
Published by Blackwell Publishing Ltd. No claim to original US government works

LCY Biotechnology Holding, Inc.
Ex. 1025
Page 4 of 11

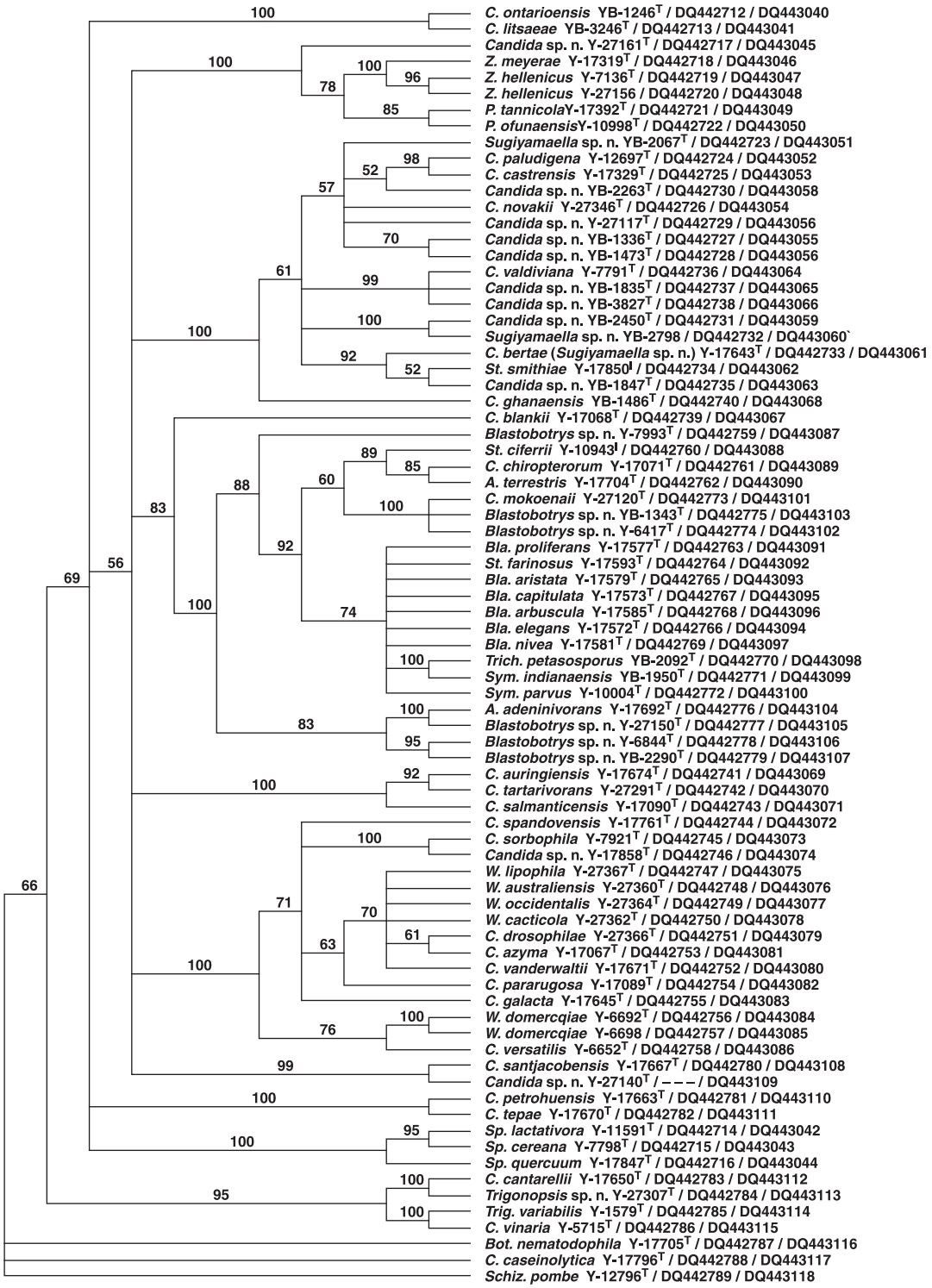

Fig. 2. Bootstrap consensus tree of species in the the *Trichomonascus*, *Wickerhamiella* and *Zygoascus* species complex from maximum parsimony analysis of combined sequences from MtSm and COXII. The analysis gave two most parsimonious trees. Tree length = 4553, consistency index (CI) = 0.302, retention index (RI) = 0.698, rescaled consistency index (RC) = 0.211, parsimony-informative characters = 681. *Schizosaccharomyces pombe* was the outgroup species in the analysis, and bootstrap values ≥50% are given at branch nodes based on 1000 replications. For each species, GenBank accession numbers follow strain numbers, with MtSm preceding COXII.

Downloaded from https://academic.oup.com/femsyr/article/7/1/141/492833 by The University of Texas at Austin user on 03 July 2024

© 2006 Federation of European Microbiological Societies
Published by Blackwell Publishing Ltd. No claim to original US government works

LCY Biotechnology Holding, Inc.
Ex. 1025
Page 5 of 11

Fig. 3. Phylogenetic tree of the *Trichomonascus–Wickerhamiella–Zygoascus* species complex represented by one of two most parsimonious trees derived from maximum parsimony analysis of combined sequences of LSU, MtSm and COXII. Tree length = 8633, consistency index (CI) = 0.349, retention index (RI) = 0.699, rescaled consistency index (RC) = 0.244, parsimony-informative characters = 1499. Bootstrap values of ≥50% are given at nodes based on 1000 replications. *Schizosaccharomyces pombe* served as the outgroup species.

Downloaded from https://academic.oup.com/femsyr/article/7/1/141/492833 by The University of Texas at Austin user on 03 July 2024

© 2006 Federation of European Microbiological Societies
Published by Blackwell Publishing Ltd. No claim to original US government works

LCY Biotechnology Holding, Inc.
Ex. 1025
Page 6 of 11

polytomies. Trees from MtSm and COXII, when analyzed separately, were nearly identical. When MtSm and COXII were combined, bootstrap support for deeper lineages increased only slightly, but no conflicts were apparent. The greatest bootstrap support of basal lineages resulted from analysis of a concatenated dataset of all three gene sequences.

Species relationships on well-supported branches were congruent for all three gene trees. Trees derived from maximum parsimony analysis were essentially identical to trees derived from neighbor-joining analysis with the Kimura 2-parameter distance correction, but bootstrap support from neighbor-joining trees was generally greater. The only conflict detected among gene trees was that of bootstrap support for the *Stephanoascus smithiae* clade. LSU analysis gave relatively weak support, with members of this group separated into three subclades. From combined MtSm–COXII analysis, the clade showed 100% bootstrap support. Concatenation of the three genes gave less than 50% support to the clade when analyzed by maximum parsimony and 56% from neighbor-joining. When the dataset included only the *Stephanoascus smithiae* and *Trichomonascus* clades, bootstrap support for the *Stephanoascus smithiae* clade increased to 75% following maximum parsimony analysis. The results were the same whether the outgroup selected was *Schizosaccharomyces pombe* or *Zygoascus hellenicus*, which is more closely related to the ingroup.

The preceding analyses have demonstrated taxonomic heterogeneity among several of the phylogenetically defined clades. Most notable is the *Trichomonascus* clade, which contains two species of *Stephanoascus* as well as species from the anamorphic genera *Arxula*, *Blastobotrys*, *Candida* and *Sympodiomyces*. Each of these clades will be discussed with proposals for reconciling species classification with phylogenetic circumscription.

Sporopachydermia clade

The three species assigned to *Sporopachydermia* represent a small, strongly supported clade that is basal to the other ascosporic clades included in this analysis.

Zygoascus clade

The two species presently assigned to *Zygoascus*, which are heterothallic and have only been isolated as haploid mating types, are quite closely related, as was demonstrated from comparisons of nuclear DNA reassociation (Smith *et al.*, 2005). Each of the species has two subpopulations, with the subpopulations for each species showing *c.* 70% nuclear DNA relatedness. Subpopulations of *Z. hellenicus* were accorded the anamorphic names *Candida steatolytica* var. *steatolytica* and *C. steatolytica* var. *inositophila*, whereas subpopulations of *Z. meyerae* were named *C. hellenica* var. *hellenica* and *C. hellenica* var. *acidophila*. The two strains of *Z. hellenicus* included in the present study represent var. *steatolytica* and showed no nucleotide differences in D1/D2, MtSm or COXII. The earlier demonstration from D1/D2 LSU analysis (Kurtzman & Robnett, 1998), showing that *Pichia ofunaensis* and *P. tannicola* are members of the *Zygoascus* clade, has been confirmed in the present analysis. For this reason, it is proposed that these two species be transferred to the genus *Zygoascus* as the following new combinations.

(1) *Zygoascus ofunaensis* (Makiguchi & Y. Asai) Kurtzman & Robnett comb. nov. Basionym: *Hansenula ofunaensis* Makiguchi & Y. Asai. *J Gen Appl Microbiol* **22**, 200, 1976. Synonym: *Pichia ofunaensis* (Makiguchi & Y. Asai) Kurtzman (1996).

(2) *Zygoascus tannicolus* (F.H. Jacob) Kurtzman & Robnett comb. nov. Basionym: *Pichia tannicola* F.H. Jacob. *Bull Soc Mycol France* **85**, 111, 1969. Synonym: *Pichia abadieae* F.H. Jacob (1969).

With the assignment of the preceding two species to *Zygoascus*, the genus description requires emendation. *Zygoascus* M. Th. Smith emend. Kurtzman & Robnett: Asci may be free, and conjugated or unconjugated, or formed on hyphae following conjugation of opposite mating types. Asci may be persistent or deliquescent, and form one to four hemispheroidal, subspherical or hat-shaped ascospores. Asexual reproduction is by multilateral budding and formation of pseudohyphae. True hyphae may also be formed and may produce blastoconidia. Sugars are fermented and nitrate is assimilated by some species.

Sugiyamaella clade

Stephanoascus smithiae and related species form a clade that is phylogenetically well separated from *Stephanoascus ciferrii*, the type species of *Stephanoascus*, as well as from *Stephanoascus farinosus*. Other members of the clade include 12 species of *Candida* as well as three undescribed ascosporic species. Bootstrap support for this clade is 100% from combined MtSm–COXII sequence analysis, but under 50% from LSU analysis. Nonetheless, there is no conflict between the two datasets for placement of well-supported species, suggesting that this clade represents a natural group. As will be discussed below, the genus name *Stephanoascus* is now a synonym of *Trichomonascus*, and a new genus is proposed for *Stephanoascus smithiae* (designated type species) and related ascosporic species.

Sugiyamaella Kurtzman & Robnett gen. nov.

Asci globosae vel ellipsoidae cum cellula apicali aut tuberculo, singuli, persistentes vel deliquenscentes tarde, uni- ad quadrispori, exorientes hyphae copulantibus. Ascosporae

Downloaded from https://academic.oup.com/femsyr/article/7/1/141/492833 by The University of Texas at Austin user on 03 July 2024

© 2006 Federation of European Microbiological Societies
Published by Blackwell Publishing Ltd. No claim to original US government works

LCY Biotechnology Holding, Inc.
Ex. 1025
Page 7 of 11

semiglobosae, ellipsoidae, petasiformes aut bacilliformis. Cellulae vegetativae globosae aut elongatae, gemmatione multilaterali propagantes; blastoconidia e cellulis conidiogenis denticulatis oriuntur. Pseudohyphae et hyphae septatae fiunt. Sacchara fermentantur aut non fermentantur. Saccharas assimilantur; amylum solubile assimilatur raro. Genus novum a generaibus aliis sequentibus nucleotiditis 26S rRNA gene, mitochondrial submonas parvus rRNA gene et cytochrome oxidase II gene distinguenda. Species typica *Sugiyamaella smithiae* (Giménez-Jurado) Kurtzman & Robnett comb. nov.

Description of *Sugiyamaella* Kurtzman & Robnett gen. nov.

Asci are globose to ellipsoidal with an apical cell or with a short protuberance. Asci arise singly on hyphae of diploid strains or following conjugation of complementary mating types. Asci form one to four ascospores, and ascus walls are usually persistent, but may deliquesce slowly. Ascospores are hemispherical, forming a hat-like shape, somewhat ellipsoidal or rod-shaped. Cell division is by multilateral budding and through blastoconidium formation, often on denticulate conidiogenous cells. Pseudohyphae and true hyphae are commonly formed. Sugars may or may not be fermented. A variety of sugars and other carbon sources are assimilated, but soluble starch is rarely utilized. Although a key to genera is provided, the most reliable means for recognizing species assigned to *Sugiyamaella* is from gene sequence comparisons.

Etymology: The genus *Sugiyamaella* is named in honor of Dr Junta Sugiyama, Professor, University of Tokyo, Japan, for his outstanding research in mycology, which has ranged from conventional studies to molecular phylogeny.

(1) *Sugiyamaella smithiae* (Giménez-Jurado) Kurtzman & Robnett comb. nov. Basionym: *Stephanoascus smithiae* Giménez-Jurado. *Syst Appl Microbiol* **17**, 240, 1994. Synonym and anamorphic state: *Candida edax* van der Walt & Nel (1968).

The remaining three ascosporic species in the *Sugiyamaella* clade, which are represented by NRRL YB-2067, NRRL YB-2798 and NRRL Y-17643, will be described in a publication now in preparation, along with the presently undescribed *Candida* species included in this clade.

Wickerhamiella clade

The type species of *Wickerhamiella*, *W. domercqiae*, and the anamorphic species *Candida versatilis*, represent members of a subclade that is basal to more recently described species of the genus (Lachance *et al*., 1998, 2000). The extent of divergence between members of the two subclades is similar to the divergence seen between subclades of the genus *Hanseniaspora* (Kurtzman & Robnett, 2003). Discovery of additional species in both *Wickerhamiella* and *Hanseniaspora* will help determine if each represents a single genus or two closely related genera. Divergence between NRRL Y-6692 and NRRL Y-6698, the two known strains of *W. domercqiae*, is approaching that of independent species.

Trichomonascus clade

The *Trichomonascus* clade (Fig. 3) is well supported from multigene sequence analysis (100%). This clade includes the ascosporic genera *Trichomonascus* and *Stephanoascus*, as well as species from the anamorphic genera *Arxula*, *Blastobotrys*, *Candida* and *Sympodiomyces*. The various genera were originally described from what appeared to be unique morphology. Asci of *Trichomonascus* form terminally on hyphae, with ascospore formation initiated after a trichogyne-like hypha, which arises from the hyphal cell supporting the ascus, extends and fuses with the terminus of the ascus. Asci of *Stephanoascus* usually arise directly from hyphae and are globose with a small, sterile apical cell (Smith & de Hoog, 1998). The close relatedness of these two genera was only recognized from D1/D2 sequence analysis of the recently described *Trichomonascus petasosporus* (Kurtzman, 2004).

The dimorphic, asexual genus *Blastobotrys* was originally described as a hyphomycete, but it was placed in the Saccharomycetales following analysis of D1/D2 sequences (Kurtzman & Robnett, 1995). The close relatedness of *Blastobotrys* with *Arxula*, *Sympodiomyces* and several *Candida* species was recognized from D1/D2 sequence analysis, but resolution from this partial gene sequence was insufficient to determine whether the genera were phylogenetically distinct. Data from the present study show that the three genera, along with several species of *Candida*, represent members of the same clade. Earlier described *Blastobotrys* species form a subclade with *Stephanoascus farinosus*. Some of the *Blastobotrys* species, such as *Bla. aristata* and *Bla. capitulata*, form blastoconidia with elongated setae, giving cells the appearance of long, slender spermatozoa (de Hoog & Smith, 1998). These cells are not produced by all members of the subclade, but they are found in cultures of *Candida mokoenaii* and *Blastobotrys* sp. n. NRRL YB-1343, which occur in an adjacent subclade.

The genus *Sympodiomyces*, in contrast to *Blastobotrys*, was initially recognized as a yeast (Fell & Statzell, 1971). The three known species form true hyphae that give rise to sympodially formed clusters of blastoconidia, which arise on denticles similar to those formed by *Blastobotrys*. LSU, MtSm and COXII sequences were not determined in the present study for the recently described *Sympodiomyces attinorum* (Carreiro *et al*., 2004), but the D1/D2 tree included with the description places *Sympodiomyces attinorum* as a sister to *Sympodiomyces parvus*. The two described species of *Arxula*, which occur in separate subclades of the

Downloaded from https://academic.oup.com/femsyr/article/7/1/141/492833 by The University of Texas at Austin user on 03 July 2024

© 2006 Federation of European Microbiological Societies
Published by Blackwell Publishing Ltd. No claim to original US government works

LCY Biotechnology Holding, Inc.
Ex. 1025
Page 8 of 11

phylogenetic tree (Fig. 3), also form blastoconidia on denticles, but with less clustering than seen in the previous two genera (Smith, 1998). Blastoconidium formation by *Candida chiropterorum* and *C. mokoenaii* is similar to what is seen in the preceding species, but not as pronounced as in *Blastobotrys*. Consequently, it appears that one of the primary reasons for placement of the preceding species in four separate anamorphic genera resides in the perceived subtleties of blastoconidium formation. Interestingly, despite the variation in ascus formation and blastoconidiophore development shown by the preceding species, the clade appears to be physiologically different from other yeasts, because all species tested grow on most of the compounds in a panel composed of hexadecane, glycine, uric acid, adenine, isobutanol, leucine, isoleucine and putrescine (Middelhoven & Kurtzman, 2003).

From our gene sequence analyses, the *Trichomonascus* clade is a single, phylogenetically circumscribed taxonomic group that can be represented by a single ascosporic genus and a single anamorphic genus. Of the two ascosporic genera in this clade, *Trichomonascus* was described in 1947 (Jackson, 1947) and has taxonomic priority over *Stephanoascus*, which was described in 1976 with *Stephanoascus ciferrii* as the type species (Smith *et al*., 1976). The anamorphic states of this clade are characterized by noticeably denticulate conidiophores, and the species assimilate a number of unusual compounds, which phenotypically separates them from typical species of the anamorphic genus *Candida*. The genus *Blastobotrys*, type species *Bla. nivea*, was described in 1967 (von Klopotek, 1967) and has taxonomic priority over *Sympodiomyces*, type species *Sympodiomyces parvus* (Fell & Statzell, 1971) and *Arxula*, type species *A. terrestris* (van der Walt *et al*., 1990). On the basis of taxonomic priorities, the following new combinations are proposed for *Trichomonascus* and its anamorphic genus *Blastobotrys*.

(1) *Trichomonascus ciferrii* (M. Th. Smith, van der Walt & E. Johannsen) Kurtzman & Robnett comb. nov. Basionym: *Stephanoascus ciferrii* M. Th. Smith, van der Walt & E. Johannsen. *Antonie van Leeuwenhoek* **42**, 125, 1976. Synonyms: *Candida ciferrii* Kreger-van Rij (1965), *Sporothrix catenata* de Hoog & Constantinescu (1981), *Candida mucifera* Kocková-Kratochvílová & Sláviková (1988).

(2) *Trichomonascus farinosus* (de Hoog, Rantio-Lehtimäki & M. Th. Smith) Kurtzman & Robnett comb. nov. Basionym: *Stephanoascus farinosus* de Hoog, Rantio-Lehtimäki & M. Th. Smith. *Antonie van Leeuwenhoek* **51**, 102, 1985. Synonym: *Blastobotrys farinosus* de Hoog, Rantio-Lehtimäki & M. Th. Smith (1985).

(3) *Blastobotrys adeninivorans* (Middelhoven, Hoogkamer-Te Niet & Kreger-van Rij) Kurtzman & Robnett comb. nov. Basionym: *Trichosporon adeninovorans* Middelhoven, Hoogkamer-Te Niet & Kreger-van Rij. *Antonie van Leeuwenhoek* **50**, 373, 1984. Synonym: *Arxula adeninovorans* (Middelhoven, Hoogkamer-Te Niet & Kreger-van Rij) van der Walt, M. Th. Smith & Y. Yamada (1990).

(4) *Blastobotrys attinorum* (Carreiro, Pagnocca, Bacci, Lachance, Bueno, Hebling, Ruivo & Rosa) Kurtzman & Robnett comb. nov. Basionym: *Sympodiomyces attinorum* Carreiro, Pagnocca, Bacci, Lachance, Bueno, Hebling, Ruivo & Rosa. *Int J Syst Evol Microbiol* **54**, 1893, 2004.

(5) *Blastobotrys chiropterorum* (Grose & Marinkelle) Kurtzman & Robnett comb. nov. Basionym: *Candida chiropterorum* Grose & Marinkelle. *Mycopath Mycol Appl* **36**, 227, 1968.

(6) *Blastobotrys indianaensis* (Kurtzman) Kurtzman & Robnett comb. nov. Basionym: *Sympodiomyces indianaensis* Kurtzman. *Antonie van Leeuwenhoek* **85**, 302, 2004.

(7) *Blastobotrys mokoenaii* (Mokwena, Jansen van Rensburg & Myburgh) Kurtzman & Robnett comb. nov. Basionym: *Candida mokoenaii* Mokwena, Jansen van Rensburg & Myburgh. *Antonie van Leeuwenhoek* **77**, 44, 2000.

(8) *Blastobotrys parvus* (Fell & Statzell) Kurtzman & Robnett comb. nov. Basionym: *Sympodiomyces parvus* Fell & Statzell. *Antonie van Leeuwenhoek* **37**, 362, 1971.

(9) *Blastobotrys terrestris* (van der Walt & E. Johannsen) Kurtzman & Robnett comb. nov. Basionym: *Trichosporon terrestre* van der Walt & E. Johannsen. *Antonie van Leeuwenhoek* **41**, 361, 1975. Synonyms: *Geotrichum terrestre* (van der Walt & E. Johannsen) Weijman (1979), *Arxula terrestris* (van der Walt & E. Johannsen) van der Walt, M. Th. Smith & Y. Yamada (1990).

With the transfer of species to *Trichomonascus* and *Blastobotrys* from other genera, the following emended genus descriptions are provided.

Trichomonascus H.S. Jackson emend. Kurtzman & Robnett

Species may or may not be mycoparasitic. Asci form terminally on hyphae, and a small hypha arises from the cell, supporting the young ascus, and extends until the tip fuses with the top of the ascus or with a small apical cell on the ascus. Alternatively, asci form on lateral outgrowths that arise between two conjugating hyphal cells. These asci bear a small apical cell but do not produce a small fusion hypha. Asci are persistent and form two to four ascospores that may be bacilliform, hemispheroidal or hat-shaped. Vegetative reproduction is by multilateral budding, formation of blastoconidia on denticulate conidiophores, and by hyphae and pseudohyphae. Sugars may be fermented, and nitrate is assimilated by some species. Anamorph genus: *Blastobotrys*.

Blastobotrys von Klopotek emend. Kurtzman & Robnett

Colonies are white to cream-colored, sometimes slightly glistening and butyrous, but often dull, powdery, and

Downloaded from https://academic.oup.com/femsyr/article/7/1/141/492833 by The University of Texas at Austin user on 03 July 2024

© 2006 Federation of European Microbiological Societies
Published by Blackwell Publishing Ltd. No claim to original US government works

LCY Biotechnology Holding, Inc.
Ex. 1025
Page 9 of 11

mycelial. Reproduction is usually by multilateral budding and less commonly by arthroconidia formed by disarticulation of hyphae. Blastoconidia are commonly formed on denticles that may occur singly on hyphae or in sympodially arranged clusters at the ends of conidiophores. Primary conidia may produce secondary conidia. Conidia may develop long setae. Hyphae and pseudohyphae are usually abundantly present. Sugars may be fermented and nitrate may be assimilated. Teleomorph genus: *Trichomonascus*.

As shown in Fig. 3, *Sugiyamaella*, *Trichomonascus*, *Wickerhamiella*, *Zygoascus* and related anamorphs form a clade with high bootstrap support (99%) and appear to represent a family. A multigene dataset with a larger number of species will be required to determine if taxa basal to this clade should be included in the same family. Because of taxonomic priority, *Trichomonascus* is the type genus of the proposed new family.

Trichomonascaceae Kurtzman & Robnett fam. nov.

Cellulae globosae vel cylindricae, gemmatione multilaterali propagantes; pseudohyphae et hyphae septatae praesentes. Asci ovoidei vel elongati, persistentes vel deliquescens yardae, 1–4 spori; ascosporae ellipsoideae, petasiformes aut bacilliformis. Familia nova sequentibus nucleotiditis 26S rRNA gene, mitochondrial submonas parvus rRNA gene et cytochrome oxidase II gene distinguenda. Genus typicus: *Trichomonascus* H.S. Jackson. *Mycologia* **39**, 712, 1947.

Trigonopsis clade

The species *Candida cantarellii*, *C. vinaria* and *Candida* sp. n. NRRL Y-27307 form a well-supported clade with *Trigonopsis variabilis* when analyzed with any of the gene sequences used in the present study. The genus *Trigonopsis* has just one assigned species, *Trig. variabilis*, which is characterized by budding cells with a triangular shape. Budding cells in the same culture may also be ellipsoidal, tetrahedral or rhomboidal, and some strains produce few or none of the triangular cells (Yarrow, 1998b). Should *Trig. variabilis* be reassigned to *Candida* or should the *Candida* species be assigned to *Trigonopsis*? Division of *Candida* into a large number of monophyletic genera based on phylogenetic analysis has had little appeal to yeast taxonomists, because most of these genera would be unrecognized from phenotype. Nonetheless, *Trigonopsis* is so well known, and the clade is so well supported, that it would seem reasonable to recognize this unique clade by retaining the genus name *Trigonopsis*. For this reason, the following new combinations are proposed, with recognition that their placement in *Trigonopsis* relies on gene sequence analysis rather than unique phenotype. However, absence of genus-specific phenotypes is becoming increasingly common for many phylogenetically defined yeast genera. Interestingly, strains of the four species in this clade, with the exception of one strain of *Trig. variabilis*, have all been isolated from grape must (Yarrow, 1998b; Meyer *et al*., 1998).

(1) *Trigonopsis cantarellii* (van der Walt & van Kerken) Kurtzman & Robnett comb. nov. Basionym: *Torulopsis cantarellii* van der Walt & van Kerken. *Antonie van Leeuwenhoek* **27**, 210, 1961. Synonyms: *Candida cantarellii* (van der Walt & van Kerken) S.A. Meyer & Yarrow (1978), *Torulopsis vinacea* Ohara, Nonomura & Yamazaki (1964).

(2) *Trigonopsis vinaria* (Y. Ohara, Nonomura & Yunome ex M. Th. Smith) Kurtzman & Robnett comb. nov. Basionym: *Candida vinaria* Y. Ohara, Nonomura & Yunome ex M. Th. Smith. *In* J.A. von Arx (1973) Centraalbureau voor Schimmelcultures, Baarn and Delft. Progress Report 1972. *Verh K Ned Akad Wetensch, Afd Natuurk*, **61**, 59.

Placement of species in *Trigonopsis* that were previously assigned to *Candida* requires the following emendation of the genus description for *Trigonopsis* to include the presence of pseudohyphae and the absence of triangular cells. *Trigonopsis* Schachner emend. Kurtzman & Robnett: Cells are triangular, tetrahedral, rhomboidal or ellipsoidal. Reproduction is by multilateral budding or budding from the projections on triangular and tetrahedral cells. Pseudohyphae may be present but true hyphae are not formed. Sugars may be fermented, but nitrate is not assimilated.

Phenotypic separation of the *Sugiyamaella*, *Trichomonascus* and *Wickerhamiella* clades

Recognition of the *Sugiyamaella*, *Trichomonascus* and *Wickerhamiella* clades and their associated anamorphic species from the phenotypic tests generally used in yeast taxonomy is difficult because of numerous shared characters. The following key provides separation of each clade as defined from the species given in Fig. 3. Possible exceptions are *Trichomonascus farinosus* and *Blastobotrys proliferans*, both of which have strains showing a diversity of reactions on physiologic tests that has led to a variable pattern of growth reactions for each of the species (de Hoog & Smith, 1998; Smith, 1998; Smith & de Hoog, 1998). The most reliable means for clade recognition is from gene sequence comparisons, because discovery of new species with atypical growth reactions may render this key inaccurate. Nonetheless, the following key provides clade recognition for currently known species.

1a Soluble starch, L-rhamnose, D-glucosamine, erythritol and inositol are not utilized for growth – *Wickerhamiella*.

b One or more of the following are utilized for growth: soluble starch, L-rhamnose, D-glucosamine, erythritol and inositol –2.

2(1)a Growth occurs with soluble starch, with nitrate and in vitamin-free medium – *Sugiyamaella*.

© 2006 Federation of European Microbiological Societies
Published by Blackwell Publishing Ltd. No claim to original US government works

Downloaded from https://academic.oup.com/femsyr/article/7/1/141/492833 by The University of Texas at Austin user on 03 July 2024

LCY Biotechnology Holding, Inc.
Ex. 1025
Page 10 of 11

b Growth occurs with soluble starch and either with nitrate or in vitamin-free medium – *Trichomonascus*.
c Soluble starch is not utilized for growth –3.
3(2)a Growth occurs with trehalose and melezitose – *Sugiyamaella*.
b Growth occurs with trehalose, but not with melezitose – *Trichomonascus*.
c Trehalose is not utilized for growth – *Sugiyamaella*.

Conclusions

On the basis of genetic crosses and molecular genetic comparisons, it has become increasingly clear that yeast species often cannot be recognized from phenotypic characters. A similar realization has developed for circumscription of genera. Seemingly unique phenotypes may or may not represent genetic isolation. Phylogenetic analyses of gene sequences have shown that many commonly accepted genera are polyphyletic (Kurtzman & Robnett, 1998, 2003; Fell *et al.*, 2000). Perhaps most surprising in the present study was that *Trichomonascus* and *Stephanoascus* are closely related, despite marked differences in their manner of ascus formation.

Acknowledgements

We thank Don Fraser for preparation of the final figures as well as the staff of the NCAUR DNA sequencing facility. Mention of company names or trade products does not imply that they are endorsed or recommended by the US Department of Agriculture over other companies or similar products not mentioned.

References

Carreiro SC, Pagnocca FC, Bacci M Jr, Lachance M-A, Bueno OC, Hebling MJA, Ruivo CCC & Rosa CA (2004) *Sympodiomyces attinorum* sp. nov., a yeast species associated with nests of the leaf-cutting ant *Atta sexdens*. *Int J Syst Evol Microbiol* **54**: 1891–1894.

de Hoog GS & Smith MTh (1998) *Blastobotrys* von Klopotek. *The Yeasts, A Taxonomic Study*, 4th edn (Kurtzman CP & Fell JW, eds), pp. 443–448. Elsevier Science B.V, Amsterdam.

Fell JW & Statzell AC (1971) *Sympodiomyces* gen. n., a yeast-like organism from southern marine waters. *Antonie van Leeuwenhoek* **37**: 359–367.

Fell JW, Boekhout T, Fonseca A, Scorzetti G & Statzell-Tallman A (2000) Biodiversity and systematics of basidiomycetous yeasts as determined by large-subunit rDNA D1/D2 domain sequence analysis. *Int J Syst Evol Microbiol* **50**: 1351–1371.

Jackson HS (1947) *Trichomonascus*, a new genus among simple ascomycetes. *Mycologia* **39**: 709–715.

Kurtzman CP (2004) *Trichomonas petasosporus* sp. nov. and *Sympodiomyces indianaensis* sp. nov., two new members of the Saccharomycetales. *Antonie van Leeuwenhoek* **85**: 297–304.

Kurtzman CP & Robnett CJ (1995) Molecular relationships among hyphal ascomycetous yeasts and yeastlike taxa. *Can J Bot* **73**: S824–S830.

Kurtzman CP & Robnett CJ (1998) Identification and phylogeny of ascomycetous yeasts from analysis of nuclear large subunit (26S) ribosomal DNA partial sequences. *Antonie van Leeuwenhoek* **73**: 331–371.

Kurtzman CP & Robnett CJ (2003) Phylogenetic relationships among yeasts of the '*Saccharomyces* complex' determined from multigene sequence analyses. *FEMS Yeast Res* **3**: 417–432.

Lachance M-A, Rosa CA, Starmer WT, Schlag-Edler B, Barker JSF & Bowles JM (1998) *Wickerhamiella australiensis*, *Wickerhamiella cacticola*, *Wickerhamiella occidentalis*, *Candida drosophilae* and *Candida lipophila*, five new related yeast species from flowers and associated insects. *Int J Syst Bacteriol* **48**: 1431–1443.

Lachance M-A, Bowles JM, Mueller C & Starmer WT (2000) On the biogeography of yeasts in the *Wickerhamiella* clade and description of *Wickerhamiella lipophila* sp. nov., the teleomorph of *Candida lipophila*. *Can J Microbiol* **46**: 1145–1148.

Meyer SA, Payne RW & Yarrow D (1998) *Candida* Berkhout. *The Yeasts, A Taxonomic Study*, 4th edn (Kurtzman CP & Fell JW, eds), pp. 454–573. Elsevier Science B.V, Amsterdam.

Middelhoven WJ & Kurtzman CP (2003) Relation between phylogeny and physiology in some ascomycetous yeasts. *Antonie van Leeuwenhoek* **83**: 69–74.

Smith MTh, van der Walt JP & Johannsen E (1976) The genus *Stephanoascus* gen. nov. (Ascoideaceae). *Antonie van Leeuwenhoek* **42**: 119–127.

Smith MTh (1998) *Arxula* van der Walt, M.Th. Smith & Y. Yamada (1998). *The Yeasts, A Taxonomic Study*, 4th edn (Kurtzman CP & Fell JW, eds), pp. 441–442. Elsevier Science B.V, Amsterdam.

Smith MTh & de Hoog GS (1998) *Stephanoascus* M. Th. Smith, van der Walt & E. Johannsen (1998). *The Yeasts, A Taxonomic Study*, 4th edn (Kurtzman CP & Fell JW, eds), pp. 400–403. Elsevier Science B.V, Amsterdam.

Smith MTh, Robert V, Poot GA, Epping W & deCock AWAM (2005) Taxonomy and phylogeny of the ascomycetous yeast genus *Zygoascus*, with proposal of *Zygoascus meyerae* sp. nov. and related anamorphic varieties. *Int J Syst Evol Microbiol* **55**: 1353–1363.

Swofford DL (1998) *PAUP 4.0: Phylogenetic Analysis using Parsimony*. Sinauer Associates, Sunderland, MA.

van der Walt JP, Smith MTh & Yamada Y (1990) *Arxula* gen. nov. (Candidaceae), a new anamorphic, arthroconidial yeast genus. *Antonie van Leeuwenhoek* **57**: 59–61.

von Klopotek A (1967) *Blastobotrys nivea* gen. nov., sp. nov. *Archiv Mikrobiol* **58**: 92–96.

Yarrow D (1998a) Methods for the isolation, maintenance and identification of yeasts. *The Yeasts, A Taxonomic Study*, 4th edn (Kurtzman CP & Fell JW, eds), pp. 77–121. Elsevier Science B.V, Amsterdam.

Yarrow D (1998b) *Trigonopsis* Schachner. *The Yeasts, A Taxonomic Study*, 4th edn (Kurtzman CP & Fell JW, eds), p. 605. Elsevier Science B.V, Amsterdam.

Downloaded from https://academic.oup.com/femsyr/article/7/1/141/492833 by The University of Texas at Austin user on 03 July 2024

© 2006 Federation of European Microbiological Societies
Published by Blackwell Publishing Ltd. No claim to original US government works

LCY Biotechnology Holding, Inc.
Ex. 1025
Page 11 of 11